氏　　名	MANIRATANACHOTE RAWIWAN
学位の種類	博士（薬学）
学位記番号	博甲第762号
学位授与の日付	平成17年9月30日
学位授与の要件	課程博士（学位規則第4条第1項）
学位授与の題目	STUDIES ON THE MECHANISMS INVOLVED IN TROGLITAZONE-INDUCED HEPATOTOXICITY （抗糖尿病薬トログリタゾンによる肝障害のメカニズムの研究）
論文審査委員（主査）	横井　毅（医学系研究科・教授）
論文審査委員（副査）	辻　　彰（自然科学研究科・教授），中島　美紀（医学系研究科・助教授）， 加藤　将夫（自然科学研究科・助教授），鈴木　永雄（自然科学研究科・教授）

要旨

The purpose of this research thesis was to find out the mechanisms involved in troglitazone-induced hepatotoxicity. Human hepatoma cell lines treated without or with troglitazone were separated by 2-dimensional electrophoresis and subjected to amino acid sequence analysis to identify the proteins of interest. Immunoglobulin heavy chain binding protein (BiP), an abundant chaperone protein in the endoplasmic reticulum (ER), was overexpressed in hepatoma cell lines by troglitazone treatment. This study showed the important role of this chaperone protein on phenotypic change in cell viability that inhibition of BiP expression by small interference RNA rendered cells more susceptible to the toxic effects of troglitazone. These results suggest that BiP overexpression is a defense mechanism of the ER in response to troglitazone-induced toxicity. In addition, ribosomal protein P0 (P0) is also one of the targets of troglitazone. Dephosphorylation of P0, rather than its overexpression, is involved in troglitazone-induced HepG2 cell toxicity. Furthermore, an implication of autoantibody formation by the toxic effects of troglitazone was demonstrated. Aldolase B was identified as an autoantigen that reacted with antibodies in sera from two patients with type II diabetes mellitus with troglitazone-induced liver dysfunction. The titer of anti-aldolase B remained high for several weeks after stopping troglitazone administration. This evidence demonstrated that troglitazone-induced hepatotoxicity may have an immunological basis. Autoantibodies to aldolase B were also detected in sera of patients with chronic hepatitis and liver cirrhosis. These findings suggest that liver injury may cause the appearance of autoantibodies to aldolase B which may have a certain effect that aggravates the hepatitis.

概要

Troglitazone (TRO) is an early member of thiazolidinedione antidiabetic agents launched in 1997. It had been reported to produce idiosyncratic hepatotoxic effects in some individuals and was considered to withdraw from the market in 2000. The mechanism by which TRO induced toxicity is still not completely understood. This research thesis was based on an attempt to find out the mechanism involved in TRO toxicity. To investigate the involvement of proteins whose regulations correlated with the TRO-induced toxic effects, a proteomic analysis strategy was used. In addition, the suspected autoantibody production associated with troglitazone in patient sera was also determined.

[Results and Discussion]

① Chaperone protein involved in troglitazone-induced toxicity in human hepatoma cell lines

The protein expression profiles of HepG2 cells treated with various concentrations of TRO or rosiglitazone (RSG) were separated by 2-dimensional electrophoresis (2-DE). A protein spot at an approximate MW of 75 kDa and isoelectric point (pI) of 5 was increased greatly in correlation with the concentration-dependent exposure to TRO. This spot was identified as a mixture of two chaperones, immunoglobulin heavy chain binding protein (BiP or Grp78) and, to a lesser extent, protein disulfide isomerase related protein or 72 kDa endoplasmic reticulum protein (ERp72). TRO treatment elicited a dose-dependent overexpression of BiP protein as confirmed by Western blotting, whereas, RSG showed no change. BiP mRNA was also induced by TRO treatment. RSG treated HepG2 cells also induced BiP mRNA expression but at a low level, which would account for its lower toxicity compared to TRO. Unlike BiP, ERp72 protein was expressed equally in all treatments. These results suggest that the damage caused by TRO treatment was unlikely related to the increased ERp72 proficiency. In HLE cells, transfection of the small interfering RNA targeting the *BiP* gene rendered cells more susceptible to the lethality by TRO. This study suggested the crucial role of BiP overexpression in the effects of TRO exposure. TRO may serve as a stress signal to the endoplasmic reticulum, which in turn, causes the overproduction of BiP in response to cytotoxicity.

② Dephosphorylation of ribosomal P0 protein involved in troglitazone-induced HepG2 cells toxicity

HepG2 cell lysates treated with or without TRO or RSG, for 48 h were separated by 2-DE and visualized by silver staining. A protein spot of interest at MW of 34.4 and pI of 5.7 was presented on the profiles in a dose-dependent of TRO treatments. This protein was identified as 60S acidic ribosomal protein P0 (P0). However, the subsequent experiments performed by Western blot analyses with anti-ribosomal P protein antibody and real-time PCR demonstrated that P0 protein and mRNA were unlikely induced by TRO treatment. Interestingly, when 2-DE gels were subsequently subjected to Western blot analyses, at least 4 spots with the estimate pI of 5.7, 5.2, 4.8 and 4.5 were revealed. These spots were completely matched with the spots on the 2-DE gels of silver staining. The shift of its pI was considered to be dephosphorylation of P0 by TRO treatment (pI 5.7) as confirmed by using calf intestinal alkaline phosphatase as a protein phosphatase to release phosphate groups from phosphorylated residues in the P0 protein and demonstrated the same pattern. The results confirmed the possibility of dephosphorylation of this protein that associated with TRO-induced toxicity. The present research proposed that P0 protein dephosphorylation played important roles in apoptosis by TRO.

③ Detection of autoantibody to aldolase B in sera from patients with troglitazone-induced liver dysfunction

Autoantibodies in sera from patients with type II diabetes mellitus with troglitazone-induced liver dysfunction were determined. Two female patients (47- and 70-year old) ceased taking troglitazone (400 mg/day) after 23.5 and 16 weeks, respectively, due to increased serum ALT. Using 2-DE and amino acid sequence analyses, aldolase B was identified as an autoantigen that reacted with antibodies in sera from both patients. The titer of anti-aldolase B remained high for several weeks after stopping troglitazone administration. The mean reactivity of autoantibodies to aldolase B determined by ELISA with sera of patients with chronic hepatitis (n = 40) and liver cirrhosis (n = 40) was significantly higher ($p < 0.05$ and $p < 0.001$, respectively) than in healthy subjects (n = 80). These findings suggest that liver injury may cause the appearance of autoantibodies to aldolase B which may have a certain effect that aggravates the hepatitis. In addition, the anti-aldolase B titer might indicate the aggressiveness of liver dysfunction.

[Conclusions]

Troglitazone-induced toxicity in human hepatoma cell lines was a result of many cellular responses. This thesis demonstrated that an alteration of protein expression, BiP chaperone protein, and a post-translational modification, dephosphorylation of P0, were also involved in the process. In addition, the appearance of autoantibodies, aldolase B, in patient sera suggests that troglitazone-induced hepatotoxicity may have an immunological basis. To understand the mechanisms of drug-induced idiosyncratic hepatotoxicity in human, further investigations in various complex ways are required.

学位論文審査結果の要旨

本研究では、２次元プロテオミクス法を駆使して、トログリタゾンに起因する肝障害のバイオマーカーの検索と検討を行い、以下の結果を得た。第一に、ヒト肝細胞のシャペロン蛋白質であるBiPの発現量が、トログリタゾンの暴露量に此例して増えることをヒト肝由来培養細胞で見出した。Bipの発現量をsiRNAでノックダウンさせることにより、トログリタゾン由来細胞毒性が有意に高くなることを見出した。これよりBipの発現増加が細胞毒性の防御機構として働くことを示した。第二に、肝リボゾームのPO蛋白質の脱リン酸化が、トログリタゾン由来肝障害と相関して変動することを２次元プロテオミクス法で明らかにした。この脱リン酸化は、カスパーゼpathwayを介する組胞毒性と関連していることを明らかにした。第三に、トログリタゾンにより肝障害を発症した患者の血清中に、肝サイトソル蛋白質に対する自己抗体の存在を見出した。２次元プロテオミクス法により、この抗原をアルドラーゼBと同定した。この自己抗体が肝硬変や慢性肝障害患者のバイオマーカーになる可能性を示した。

以上の研究成績は、トログリタゾンによる肝障害発症に２種類のシャペロン蛋白質が関与することを初めて証明し、また、トログリタゾン由来肝障害発症患者の血清中に自己抗体を見出し、この抗体が肝障害のマーカーになる可能性を初めて示した先駆的な研究内容であり、ヒトにおける薬物誘導性の肝障害の理解に新たな進歩をもたらした点が高く評価されるので、審査委員会は本論文が博士（薬学）に値すると判断する。